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The possible protective role of pumpkin seed oil in an animal model of acid aspiration pneumonia: Light and electron microscopic study

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ABSTRACT

Aspiration pneumonitis is a common problem occurring in many clinical disorders. Pumpkin seed oil (PO) is a rich source of antioxidants. This work aimed to assess the effect of PO on the lung histopathological changes induced by acid aspiration. Forty male albino rats assigned to four groups were used. Rats of **control** group were instilled intratracheally with normal saline 2 mL/kg. **HCL** group instilled with 2 mL/kg of HCL 0.1N, pH 1.25. **PO** group received pumpkin seed oil (PO) orally (~1375 mg/kg bw/day) for 7 days. **HCL + PO** group instilled with 2 mL/kg of HCL 0.1N, pH 1.25 and received PO at the same dose of **PO** group. Lung tissue samples were processed for light, electron microscopic and immunohistochemical study using anti inducible NO synthase (iNOS). The lung of **HCL** group demonstrated thickened interalveolar septa, inflammatory cell infiltration and significant increase in the area percent of collagenous fibers and immune expression of iNOS. Ultra structurally, disrupted alveolocapillay membrane, degenerated type II pneumocytes and plentiful alveolar macrophages were evident. PO administration partially attenuated these histological and ultra structural alterations and reduced iNOS immune-expression in lung tissue. In conclusion, PO has a protective effect against HCL aspiration lung injury most probably through its antioxidant activity.

1. Introduction

The aspiration of gastric contents is a common and critical complication which occurs in a lot of clinical disorders. It is one of the major health problems that occur during the care of critically ill patients (Tasch and Stoelting, 1996). The aspiration of gastric fluid commonly occurs in patients with trauma especially that of the head. It also occurs in alcoholics and in patients with cerebrovascular accidents. Also, general anesthesia is complicated with acid aspiration in one per 2000–3000 cases receiving general anesthetics (Raghavendran et al., 2011; Marik, 2001). The aspiration of gastric contents results in acute lung injury. Several factors affect the degree of the histopathological changes induced by gastric content aspiration such as the amount of the aspirated substance, pH value and the patient reaction (James et al., 1984). Previous workers have demonstrated the histopathological changes occurring in different animal models of aspiration pneumonia such as peribronchial inflammatory cell infiltration, alveolar edema, alveolar exudate, alveolar histiocytes and interstitial fibrosis (Guzel et al., 2009). There is evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role in the pathophysiology of aspiration induced lung injury (Guzel et al., 2009; Metnitz et al., 1999).

Nitric oxide (NO) is one of the highly reactive oxygen species. It is synthesized from L- arginine by the action of NO synthase enzyme and could be originated from vascular endothelial, immune and nerve cells (Moncada, 1992; Nathan and Xie 1994). iNOS is induced by inflammatory stimuli including cytokines, bacteria, and bacterial products e.g. lipopolysaccharides (Ermert et al., 2002). It is expressed by endothelial, epithelial and inflammatory cells and produced in large quantities for extended durations (Gaston et al., 1994).

The pumpkin (Cucurbita spp.) is a traditional food which was known in Europe since the 16th century. Pumpkin seeds are considered as a suitable source of edible oil. Pumpkin seed oil (PO) has valuable nutritional benefits. It contains considerable amounts of palmitic, stearic, oleic, and linoleic fatty acids (Butinar et al., 2010; Stevenson et al., 2007). It also contains large amounts of vitamin E and tocoferol (Stevenson et al., 2007). PO was proved to be effective in the treatment of benign prostatic hyperplasia (Hong et al., 2009). It has been also reported to decrease the oxidative damage and lipid peroxidation induced by aflatoxins (Eraslan et al., 2013). The value of PO has been reported in many health problems such as cancer, hypertension, hyperlipidaemia, urinary disorders and dia-

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betes (Boaduo et al., 2014; Jian et al., 2005a; Nishimura et al., 2014; Zuhair et al., 2000). Anti-inflammatory and antibacterial activities for PO have been also reported (Hammar et al., 1999; Nawirska-Olszańska et al., 2013). It could be also used in the manufacture of pharmaceutical and cosmetic products and may play a role in disease prevention and health promotion (Siano et al., 2016).

Previous studies have reported a beneficial role of antioxidants in the prevention and management of acute lung injury (Bhatia and Moochhala, 2004; Tasaka et al., 2008). However, to our knowledge, there is no previous works which investigated the impact of PO on acid aspiration pneumonia. Also, very little is known about the beneficial role of antioxidants in acid aspiration pneumonia at the ultra structural level. Therefore, this study was performed in order to reveal the histological, immunohistochemical and ultra structural changes induced by acid aspiration on the rat lung and to investigate whether or not treatment with PO could attenuate such changes.

2. Materials and methods

2.1. Experimental animals

Forty male albino rats with average weight (180–220 g) were used in this study. The experiment was done after obtaining prior approval (R/15.08.70) from the animal ethics committee of Mansoura Faculty of Medicine (Institutional Research Board; IRB). The duration of the experiment was 7 days. The animals were kept in separate cages in average temperature (22 ± 2 °C) and average humidity (50–55%) in an adequately ventilated room. The animal were subjected to a regular 12 h light/12 h dark cycle and were allowed free access to food and water ad libitum. The animal housing and all experimental procedures were carried out in Nile Research Centre in Mansoura city, Egypt.

2.2. Animal grouping

The rats were separated into four groups; 10 animals each.

1. **Control** group: instilled intratracheally with normal saline 2 mL/kg.
2. **PO** group: instilled intratracheally with normal saline 2 mL/kg and given pumpkin seed oil (PO) once a day at a dose of (~1375 mg/kg bw/day) for 7 days (orally with aid of gastric tube).
3. **HCL** group: instilled intratracheally with HCL (HCl 0.1 N, pH 1.25) injected into the lungs in a volume of 2 mL/kg.
4. **HCL + PO** group: instilled intratracheally with HCL as **HCL** group and given PO once a day at a dose of (~1375 mg/kg bw/day) for 7 days (orally with aid of gastric tube).

2.3. Procedure: (Guzel et al., 2009)

The animals underwent anesthesia by ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/ kg) intraperitoneally and were allowed for spontaneous breathing during the experiment. The animals were put in a supine position with their limbs drawn caudally to help tracheal exposure. An anterior neck incision was done in order to expose the trachea. A direct puncture was done two to four tracheal rings below the larynx. Saline or Hydrochloric acid (HCl 0.1 N, pH 1.25) were instilled into the lungs in a volume of 2 mL/kg according to the animal group. Then, the neck incision was closed with a 6–0 Ethilon suture and the rats were put under observation till recovery from the anesthesia. After recovery, rats of **PO** and **HCL + PO** groups were given PO once daily at a dose of (~1375 mg/kg bw/day) for 7 days (orally with aid of gastric tube). At the end of the seventh day, all animals were fasted over night, then, anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Subsequently, midline sternotomy was done and lungs were exposed. From all groups; 4 randomly selected specimens from right lung (anterior, median, posterior and post caval lobes) and 2 randomly selected specimens from left lung (upper left, and lower left lobes) were obtained and processed for light and electron microscopy respectively.

3. Histological study

3.1. Light microscopic study

Paraffin serial sections (5 μm thick) were prepared and stained with Hematoxylin and Eosin stain (Drury and Walington, 1980) and Masson's trichrome stain (Jones et al., 2008)

3.2. Immunohistochemical study

Using Avidin Biotin peroxidase Complex (ABC) technique, paraffin sections of the lung were stained with anti inducible nitric oxide (anti-iNOS) antibody (Hsu et al., 1981).

3.2.1. Technique

First, endogenous peroxidase activity was inhibited by incubating the sections with 3% H2O2 in distilled water for 30 min. Subsequently, the sections were washed in tap water for 30 min followed by washing in distilled water for 10 min. To block non specific binding of antibodies, the sections were incubated with normal goat serum (X 0907; Dako, Carpinteria, California, USA) with PBS, diluted 1:4. After that, sections were incubated with anti inducible nitric oxide synthase (anti-iNOS) antibody (GeneTex, GTX74171) diluted 1:100 and then left at room temperature for 1 h. Then, the sections were washed in PBS 3 × 3 min and incubated with biotinylated antimouse IgG (LSAB 2 Kit; Dako), then washed in PBS 3 × 3 min and incubated with avidin–biotin–peroxidase complex solution (LSAB 2 Kit; Dako). To visualize sites of antibody binding to tissue, sections were then treated with 0.05% (w/v) 3,3′-diaminobenzidine tetrachloride (Sigma Chemicals Co., St. Louis, MO, USA) in 10 mmol Tris-buffered saline Then, Mayer's hematoxylin was used to counterstain the sections. Negative control sections were placed under the same conditions but the step of primary antibodies was omitted. Positive controls were included within the tissue sections and a positive reaction was demonstrated in the liver.

3.3. Electron microscopic study (Woods and Stirling, 2008)

Small specimens (1 × 1 × 1 mm) of the lung were kept in 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) overnight. Then specimens were post fixed in 1% osmium tetroxide for 1 h at 4C° then washed three times in phosphate buffer saline (10 min each). Semithin sections (1 μm) were cut using ultra microtome (Leica ultra cut UCT, Germany), then stained with 1% toluidine blue. Ultrathin sections (60–80 nm) were obtained and stained of 2% uranyl acetate for 10 min followed by Reynold's lead citrate for 10 min. Ultrathin sections were then examined and photographed using JEOL-JEM-100 SX transmission electron microscope in the Electron Microscopy unit, Faculty of Science, Alexandria University, Egypt.

3.4. Histomorphometry and area percent

Slides were photographed using Olympus digital camera (E420, China) installed on Olympus microscope with 0.5x photo adaptor, using objective lens x 40 (TX 31 Philippines). The resultant images were analyzed on Intel Core 13 based computer using VideoTesT

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